

Matrix Metalloproteinase-9 and Paraoxonase 1 Q/R192 Gene Polymorphisms and the Risk of Coronary Artery Stenosis in Iranian Subjects

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Purpose: To investigate the association of matrix metalloproteinase-9 (MMP-9) and paraoxonase 1 (PON1) 192 polymorphisms with susceptibility to coronary artery stenosis (CAS) and the number of diseased vessels in patients with CAS. **Methods:** The study population comprised 302 unrelated Iranian individuals, including 145 patients with CAS and 157 control subjects. Genotypes for MMP-9 and PON1 192 polymorphisms were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). **Results:** In our study, distributions of the TT genotype of MMP-9 and the RR genotype of PON1 192 were significantly higher in patients compared with healthy control

subjects ($P < 0.05$). Subsequent analysis demonstrated that a significant difference existed in the male (TT+TC vs. CC and RR+QR vs. QQ, $P < 0.01$) but not in the female. The associations of these polymorphisms with the severity of stenosis were also evaluated, which according to results distribution of MMP-9 and PON1 192 genotypes were not significantly different compared with the severity of stenosis ($P > 0.05$). **Conclusions:** The observation indicates that the polymorphisms in the MMP-9 and PON1 192 genes potentially play a role in the manifestation of coronary atherosclerosis but does not have any effect on the number of diseased vessels in Iran. *J. Clin. Lab. Anal.* 24:305–310, 2010. © 2010 Wiley-Liss, Inc.

Key words: coronary artery stenosis; matrix metalloproteinase-9; paraoxonase 1; polymerase chain reaction; restriction fragment length polymorphism

INTRODUCTION

Several studies have suggested that either paraoxonase-1 (PON1) or matrix metalloproteinases (MMPs) are associated with oxidative stress (1,2). It has been indicated that high-density lipoprotein (HDL) inhibits oxidation of LDL and thus may protect against risk of coronary artery disease (CAD) (3,4). The ability of HDL to prevent the oxidation of LDL has been attributed to several HDL-associated enzymes, namely paraoxonase (PON1) (5–7), platelet-activating factor acetyl hydrolase (8), and lecithin:cholesterol acyltransferase (9). PON1 is an enzyme with a molecular mass of 43 kDa (354 amino acids) and in serum it is exclusively located on HDL (10). PON1 has two common coding polymorphisms: a methionine-to-leucine substitution at

codon 55 (M/L55) and a glutamine-to-arginine substitution at position 192 (Q/R192). Both polymorphisms have been associated with a number of pathophysiological conditions (11). MMP9 (also known as 92-kDa type IV collagenase or gelatinase B) possesses proteolytic activity on elastin as well as other basement membrane proteins including fibronectin, laminin, and

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type IV collagen that surrounds every vascular smooth muscle cell and underlies the endothelium in the blood vessel wall (12). Studies have shown that MMP9 plays a key role in vascular smooth muscle cell migration and macrophage infiltration in atherogenesis, both of which requires degradation of the basement membrane (13,14). A number of polymorphisms in the MMP9 gene have been identified (15): -1562C/T, a CA repeat, +6C/T and the coding R279Q. In vitro experiments have showed that the T-1562 allele has higher promoter activity in driving gene expression than the C-1562 allele (16). Although epidemiologic studies suggest that several genetic variants increase the risk of CAD, the association studies that examine many polymorphisms simultaneously are required to allow reliable prediction of the genetic risk of CAD. Therefore, this study was designed as a case-control study in a sample of Iranian subjects undergoing diagnostic coronary angiography to investigate the correlation of the MMP9 C/T and PON1 Q/R192 gene polymorphisms with the incidence and severity of coronary artery stenosis (CAS).

MATERIALS AND METHODS

Study Subjects

The cases and controls were selected by simple sampling from unrelated individuals. One hundred forty-five patients (75 male, 70 female; mean age: 58.41 ± 9.12) and 157 healthy individuals (80 male, 77 female; mean age: 55.35 ± 9.43) were entered into this study. Patients were selected from Shahid Rajaee Hospital (Tehran, Iran) and Shahid Madani Hospital (Tabriz, Iran) if they had CAD, which was defined as coronary stenosis of $>50\%$ in at least one major coronary artery in the angiography performed after a clinical suspicion. Those with coronary stenosis less than 50% were excluded from the study. Severity of CAD was determined by the number of coronary arteries involved (single vessel disease, double vessel disease, and triple vessel disease).

Healthy individuals were those without history of angina pectoris who had never been treated for coronary diseases. Individuals with hepatic or renal disease, cardiomyopathy, congestive heart failure, acute myocardial infarction within the last 3 months, diabetes mellitus, hypertension, hyperlipidemia, and those who

worked with insecticides were excluded from the study. A questionnaire was completed for all patients and controls to collect data regarding their demography, medical status and history, and family history. Blood pressure, weight, and height were also recorded. This study was approved by the University Hospital Ethics Committee and written informed consent was obtained from all patients and control subjects.

Laboratory Measurements

Serum from samples were used to estimate lipid levels. Total cholesterol (TC), HDL, HDL-cholesterol, and triglyceride concentrations were calculated using Friedewald formula (17).

Determination of Genotypes

Genomic DNA was extracted from peripheral blood leukocytes by the salting-out method (18). Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) was used to determine the MMP9 and PON1 polymorphisms. PCR primers used in the current study are listed in Table 1. Genomic DNA was amplified in 50 μ l of mixture containing 300 ng of DNA template, 0.4 μ mol/l each primers, 200 μ mol/l dNTPs, 5 μ l of $10 \times$ reaction buffer, and 1.25u Taq DNA polymerase. The PCR cycling conditions were 2 min at 95°C followed by 35 cycles of 45 s at 94°C , 75 s at 65°C (MMP-9) or 45 s at 64°C (PON1), and 60 s at 72°C , and with a final extension at 72°C for 10 min. PCR products were digested for 16 h at 37°C (MMP-9) or for 8 h at 37°C (PON1) in a 15- μ l reaction containing 5 U MseI and AlwI restriction enzymes (New England Biolabs, Ipswich, MA), respectively, for the determination of MMP-9 and PON1 genotypes. The digested products were subjected to gel electrophoresis and visualized using ethidium bromide staining. The sizes of PCR products of MMP-9 and PON1 were 435 and 99 bp, respectively. The MMP9 C alleles were represented by a band of 435 bp and the T alleles were represented by two bands of 244 and 191 bp, whereas heterozygotes displayed a combination of both (435, 247, and 188 bp). For the PON1 SNP, the Q alleles were expected as a band of 99 bp, the R alleles with two bands of 65 and 34 bp, and the heterozygotes show both alleles (99, 65, and 34 bp).

TABLE 1. Sequences of PCR Primers

Gene	Polymorphism	Forward primer (5'-3')	Reverse primer (5'-3')
MMP-9	C/T	GCCTGGCACATAGTAGGCCC	CTTCCTAGCCAGCCGGGCATC
PON1	Q/R	TATTGTTGCTGTGGGACCTGAG	CACGCTAAACCCAAATACATCTC

MMP, matrix metalloproteinase; PCR, polymerase chain reaction; PON1, paraoxonase1.

All assays were conducted blindly by two researchers without the knowledge of case or control status. Additionally, approximately 10% of the samples were randomly selected and retested, and the results were 100% concordant.

Statistical Analysis

Student's *t*-test was used for comparison of age, BMI, and lipid profile in control and patient groups. Genotype frequencies were compared by the χ^2 test. Allele frequencies were determined by the gene counting method, and Hardy–Weinberg equilibrium was tested by the χ^2 test. The relationship between MMP9/PON1 genotypes and severity of disease in patient group was evaluated by the χ^2 test. All the calculations were performed using the SPSS 14 program (SPSS for Windows, Chicago, IL).

RESULTS

Table 2 summarizes the clinical profiles of all participants according to the coronary angiogram. All the baseline characteristics of the two groups are quite similar. The distributions of genotypes and alleles for MMP9 C/T and PON1 Q/R 192 in patients and control subjects are presented in Table 3. The genotype distributions satisfied the Hardy–Weinberg equilibrium. Significant differences were observed in the frequency of MMP9 and PON1 genotypes between controls and patients. Patients with CAS had a higher frequency of the TT and RR genotypes than the control group ($P < 0.05$). According to genotype data, there was a significantly higher frequency of –1562 T allele of MMP9 SNP and R allele of PON1 in patients compared with controls (Table 3; $P < 0.05$). Subsequent analysis of either gender participants shows that in males the T and R alleles frequency were significantly higher in the patient group (TT+TC vs. CC and RR+RQ vs. QQ $P < 0.01$), whereas in females, it is similar in both groups (Tables not shown). When the frequencies of genotype and alleles of MMP-9 and PON1 were compared with the number of diseased vessels, frequencies of the TT genotype and the T allele of MMP-9 were lower, and the frequency of the C allele was higher, in patients with one vessel compared with patients with two or three diseased vessels (Table 4); however, this difference was not statistically significant owing to the small number of subjects in each genotype ($P > 0.05$). PON1 gene variants were not associated with the number of diseased vessels ($P > 0.05$).

DISCUSSION

In this study, we evaluated the distribution of MMP9 C/T and PON1 Q/R192 genotypes in angiographically

TABLE 2. Classical Coronary Risk Factors and Lipid Profile in Patient and Control Subjects^a

Variable	Control group	Patient group	P-value
Age (year)	55.35 ± 9.43	58.41 ± 9.12	NS
Sex (male/female)	(80/77)	(75/70)	NS
BMI (kg/m ²)	27.24 ± 4.06	26.14 ± 4.52	NS
SBP (mm hg)	12.35 ± 1.33	12.75 ± 1.32	NS
DBP (mm hg)	7.67 ± 0.54	7.73 ± 0.34	NS
Cholesterol(mg/dl)	172.43 ± 36.76	177.12 ± 38.65	0.17
Triglyceride (mg/dl)	145.31 ± 61.54	156.23 ± 64.34	0.23
HDL-C (mg/dl)	38.44 ± 9.04	37.85 ± 9.19	NS
LDL-C (mg/dl)	94.14 ± 29.53	96.13 ± 23.13	NS
VLDL-C (mg/dl)	39/01 ± 21.17	41.12 ± 20.40	NS

$P < 0.05$ was considered statistically significant. F/M, females/males; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

^aData are presented as mean ± SD.

TABLE 3. The Genotypes Distributions of the MMPs Polymorphisms Among Controls and Patients

		Patient group <i>n</i> (%)	Control group <i>n</i> (%)	P-value
MMP-9	TT	77 (52.93)	62 (40.56)	<0.05
	TC	57 (39.65)	76 (46.25)	
	CC	11 (7.42)	19 (13.19)	
	TC+TT	134 (92.41)	138 (87.89)	
	T	211 (72.76)	200 (63.70)	
PON1	C	79 (27.24)	114 (36.30)	<0.01
	QQ	42 (28.56)	73 (45.58)	
	QR	71 (49.77)	66 (43.87)	
	RR	32 (21.67)	18 (10.55)	<0.05
	QR+RR	103 (71.03)	84 (53.50)	
	Q	155 (53.45)	212 (67.51)	
	R	135 (46.55)	102 (32.49)	

MMP, matrix metalloproteinase; PON1, paraoxonase1.

TABLE 4. MMP-9, and PON1 192 Polymorphisms Compared With the Number of Diseased Vessels in Patients

Gene	Genotype	One vessels <i>n</i> (%)	Two vessels <i>n</i> (%)	Three vessels <i>n</i> (%)
MMP-9	TT	9 (34.61)	17 (41.46)	32 (41.03)
	TC	5 (19.24)	10 (24.30)	17 (21.80)
	CC	12 (46.15)	14 (34.14)	29 (37.18)
	T	23 (44)	44 (54)	81 (64)
	C	29 (56)	38 (46)	45 (36)
	QQ	19 (33.93)	8 (23.53)	16 (29)
PON1	QR	25 (44.65)	22 (64.70)	25 (45.45)
	RR	2 (21.42)	4 (11.77)	14 (25.45)
	Q	63 (56)	38 (56)	57 (52)
	R	49 (44)	30 (44)	53 (48)

MMP, matrix metalloproteinase; PON1, paraoxonase1.

defined CAD patients and control subjects and found these polymorphisms as a risk factor for atherosclerosis. However, as the sampling method was simple, the cases and controls are not true representative of the ethnically mixed Iranian population. Accordingly, the results may not be applicable to some Iranian ethnical groups. Our study shows that the frequency of the -1562 T allele of MMP-9 and the 192 R allele of PON1 in CAD patients were significantly higher than that in healthy controls.

Transfection experiments and DNA-protein interaction assays indicated that the T allele had higher activity (19,20). Several studies indicate that the T allele has also been associated with complicated coronary lesions, and carriers of the T allele had greater levels of MMP9 mRNA and protein, and stiffer large arteries (20,21). MMP9 knockout studies in mice have also demonstrated a role of MMP9 in the development of atherosclerosis (22). Compared with MMP9 wild-type mice, MMP9-deficient mice have fewer and smaller atherosclerotic lesions (23). Smooth muscle cell migration into the intima is reduced in MMP9-deficient mice (14,24) and atherosclerotic lesions in MMP9-deficient mice contain fewer macrophages (23). Thus, increased vascular smooth muscle migration and macrophage infiltration are likely a mechanism that, at least partly, explains the finding of increased coronary atherosclerosis in carriers of the MMP9 high expression T-1562 allele in humans and this mechanism may explain why in our study the MMP9 T allele has been found to be present at an increased frequency in CAD.

There is a 10- to 40-fold difference in serum paraoxonase activity among individuals, as assessed by the enzyme's ability to hydrolyze exogenous, non physiological substrates. The glutamine (Q) to arginine (R) substitution at position 192 independently influence PON1 activity and have been defined as the part of the molecular basis for this inter-individual variability (25). It has been shown that the PON1 R allozyme is less efficient at retarding the oxidation of LDL than is the Q allozyme because of the decreased hydrolysis of lipid peroxides by the R allozyme (26,27). This finding may explain why in our study the PON1 R allele has been found to be present at an increased frequency in CAD.

Moreover, this study demonstrated that without considering other risk factors, PON1 192 and MMP9 polymorphisms are not related to severity of CAS. Although this finding seems a little unusual, it must be noted that polymorphisms consist only a part of variations of PON1 enzymatic activity and beside genetic factors, enzyme activity was influenced by acquired and environmental factors, such as diet, life style (smoking, alcohol drinking,...), environmental chemical material, age, sex and other different factors (28).

Previous studies have investigated the association between MMP9 and PON1 192 polymorphisms and CAD. Evidence from genetic epidemiological studies indicates that individuals carrying the T-1562 allele are predisposed to the development of coronary atherosclerosis that results in significant coronary stenosis (15,16). In addition, an autopsy study by Pollanen et al. shows that carriers of the T-1562 allele have larger atheromas than non-carriers, and this difference is more pronounced in older people (29). Transfection experiments and DNA-protein interaction assays indicated that the T allele had higher activity (29,30). Several studies indicate that the T allele has also been associated with complicated coronary lesions, and carriers of the T allele had greater levels of MMP9 mRNA and protein, and stiffer large arteries (30,31).

In 81 stroke patients who were compared with 2553 control subjects, PON1R was shown to be an independent risk factor for stroke (32). Similarly, in a case-control study of 139 CAD patients and 119 control subjects, RR genotype not only defined as an independent risk factor for CAD but also as a factor that related with the severity of disease (33). In accordance with our study, in these studies, the QQ genotype was the most common genotype among control subjects, whereas QR genotype was the most common among CAD patients. However, in the study by Pasdar et al. that was performed in 397 stroke patients and 400 control subjects, no association could be found between stroke and PON1 gene polymorphisms, suggesting no association between this disease and variations in PON1 alleles (34). Finally, in the study by Flekac et al. who compared diabetic patients (some of this patients had macrovascular complications) and control subjects found a statistically significant difference in the distribution of PON1 genotypes or alleles between CAD patients and controls (35); but, on the contrary of our study, homozygosity of PON1 for the Gln allele (QQ) showed lower antioxidant activity and found to be an independent risk factor for CHD.

First possible explanations of these discrepancies may be the variability of population selection and study design. Furthermore, large differences between ethnic populations are known in the PON1 genotype distribution, which may be the other reason for differences among studies (36). Finally, it must be noted that, in general, the RR genotype has high paraoxonase activity and the QQ genotype has low activity (35). However, the antioxidant capacity of PON1 is not always identical to PON1 enzymatic activity and the PON1 genotype, which may cause the controversial association of PON1 with CAD. Although it has been shown that the RR genotype is less efficient at hydrolyzing LDL peroxides and this finding may explain why in this study

and some other studies the paraoxonase RR genotype has been found at an increased frequency in patient group (37,38), recently it has been defined that the PON1-192Q binds HDL with a 3-fold lower affinity than the R isozyme and consequently exhibits significantly reduced stability, lipolactonase activity, and macrophage cholesterol efflux (38). It has also been shown that impairing the lactonase activity of PON1, through mutations of its catalytic dyad, diminishes PON1's ability to prevent LDL from oxidation (39) and the findings of the recent clinical study complete these results, demonstrating that individuals with the arginine (R) mutation at position 192 have higher serum levels of PON1 activity (40). These studies indicate that despite partially expanding researches, yet the mechanism of antioxidant activity of PON1 and spatially this fact that which of the PON1 genotypes has higher antioxidant activity is not well defined and this issue may be one of the other probable differences in the results of different studies.

There were several limitations for our study. First, this was a small study, and these observations must be confirmed in a larger sample of patients. Nevertheless, a large-scale prospective study should be launched to confirm the associative effect of these polymorphisms with CAS patients. Second, we analyzed only two polymorphisms in most of the genes, which could have missed an association that was to a specific polymorphism. It will be interesting also to see whether these polymorphisms have a role in the manifestation and development of other CAD such as myocardial infarction, coronary aneurysm, etc...

The results of this study can potentially help in the identification of people at higher risk of development of coronary heart disease. It might be possible to develop a genetic risk calculator using a profile of known genetic markers, including those described here, to determine each individual's genetic risk. If the risk is high, employment of strict atherosclerosis preventive measures starting from the young age can theoretically decrease the incidence and prevalence of this disease.

In conclusion, the MMP9 T allele and PON1 R allele are associated with CAS. Atherosclerosis is a complex disease that depends on multiple factors, including genetic, dietary, environmental, and pharmacological factors (41). In this study, we evaluated only one factor in the pathogenesis of this disease i.e. the polymorphism of a single related gene that is one of several factors involved in this complex disease and as there are some different factors to cause this disease as well as its severity, it is suggested that although in our study PON1RR genotype and MMP9 TT genotype has been found as a risk factor for stenosis but there are,

probably, more important factors rather than polymorphism, involved in the severity of the disease.

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